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Virulence gene repression promotes *Listeria monocytogenes* systemic infection

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ABSTRACT

The capacity of bacterial pathogens to infect their hosts depends on the tight spatiotemporal regulation of virulence genes. The *Listeria monocytogenes* (*Lm*) metal efflux pump repressor CadC is highly expressed during late infection stages, modulating lipoprotein processing and host immune response. Here we investigate the potential of CadC as broad repressor of virulence genes. We show that CadC represses the expression of the bile salt hydrolase impairing *Lm* resistance to bile. During late infection, in absence of CadC-dependent repression, the constitutive bile salt hydrolase expression induces the overexpression of the cholic acid efflux pump MdrT that is unfavorable to *Lm* virulence. We establish the CadC regulon and show that CadC represses additional virulence factors activated by σ^B during colonization of the intestinal lumen. CadC is thus a general repressor that promotes *Lm* virulence by down-regulating, at late infection stages, genes required for survival in the gastrointestinal tract. This demonstrates for the first time how bacterial pathogens can repurpose regulators to spatiotemporally repress virulence genes and optimize their infectious capacity.

Introduction

Listeria monocytogenes (Lm) is a major intracellular foodborne bacterial pathogen which causes listeriosis, a human systemic infection.[1] Among zoonotic diseases under EU-surveillance, listeriosis is the most severe.[2] *Lm* has the capacity to colonize various niches, from inert and organic matrices to the intestinal lumen where it competes with resident microbiota, translocates across the epithelium, multiplies in phagocytic and non-phagocytic cells and disseminates *via* the blood.[1,3] *Lm* can grow at temperatures ranging from 0 to 45ºC, under high osmolarity and acidic conditions, which it may encounter in the environment, in the food chain, as well as in the host. During infection, *Lm* faces proteolytic enzymes, acidic environment, high osmolarity and bile salts.[4]

Bile salts are amphipathic molecules synthesized from cholesterol in the liver and secreted into the small intestine from the gall bladder.[5] They are

major bile components able to degrade lipid-containing membranes and represent a key challenge to bacterial survival in the gastrointestinal tract.[6] Bacterial tolerance to bile salts is closely related to the activity of bile salt hydrolases (BSH) that catalyze the hydrolysis of glycodeoxy-cholate- (GDCA) and taurodeoxycholate- (TDCA) conjugated bile salts, thereby impairing bile toxicity toward bacteria. BSH is required for *Lm* resistance to bile and virulence.[7] Deconjugation of bile acids by BSH induces the release of free cholic acids (CA), that are exported by *Lm* through the MdrT efflux pump. *mdrT* is controlled by BrtA, a bile sensor which loses the ability to repress *mdrT* in the presence of CA.[8]

To adapt and resist to the host environment, *Lm* evolved an arsenal of mechanisms that must be spatially and timely regulated.[9] PrfA, the major *Lm* virulence regulator, and σ^B, a general stress responsive sigma factor, were shown to control

bsh expression and *Lm* tolerance to bile.[7,10,11] CadC is the transcriptional regulator of CadA, an efflux pump conferring cadmium resistance.[12] We previously showed that during *in vivo* infection, *Lm* uses CadC to directly repress the expression of LspB (lipoprotein signal peptidase B), thus avoiding the exposure of the lipoprotein LpeA to the host immune system, impairing inflammatory cytokine expression and ultimately promoting intramacrophage survival and virulence.[12]

Results

CadC controls BSH activity and Lm resistance to bile salts

We hypothesized that CadC could be a broader regulator required for *Lm* survival and virulence. We investigated the potential involvement of CadC in the *Lm* resistance to different stresses that it could encounter in environmental and host conditions. In particular, we assessed the growth of the Δ*cadC* mutant[12] at pH 5.5 or in the presence of high concentrations of either NaCl or lysozyme. As shown in Figure 1a, no significant difference was observed between the growth of the wild type and the mutant strains in BHI broth at pH 5.5 or containing 5% NaCl. Similarly, no growth defect was detected in BHI broth containing 50 μg/ml of lysozyme (Figure 1b). As expected, we observed a significant decrease in the survival of the lysozyme-hypersensitive Δ*pgdA* mutant used as positive control (Figure 1b).[13] These data demonstrate that CadC has no role in the resistance to these stresses.

During infection, *Lm* has to resist to host bile.[6] This resistance is mainly promoted by the BSH that catalyzes the deconjugation of glyco- (GDCA) and tauro- (TDCA) conjugated bile salts under low oxygen levels.[7,10] We analyzed the impact of *cadC* deletion on BSH activity and resistance to bile acids. The BSH activity of the WT, Δ*cadC* and Δ*cadC*+*cadC* strains was evaluated by patch inoculation onto Man-Rogosa -Sharpe (MRS) medium supplemented with increasing concentrations of GDCA or TDCA and grown under microaerophilic conditions. The WT strain exhibited the formation of a classical white area corresponding to precipitated bile acids,[14] confirming the presence of BSH activity (Figure 1c,d), whereas the nonpathogenic *L. innocua*, that lacks *bsh*,[7] was nearly

incapable of precipitating GDCA (Figure 1c). As compared to the WT strain, the Δ*cadC* mutant displayed a more pronounced precipitate, a phenotype that was reverted in the Δ*cadC*+*cadC* strain. In addition, the Δ*cadC* strain was able to precipitate bile acids at concentrations where the WT was unable to deconjugate them (Figure 1c,d), indicating that CadC plays a role in *Lm* BSH activity. The Δ*cadAC* double mutant behaved as the Δ*cadC* single mutant (Figure 1c), indicating that the role of CadC in *Lm* BSH activity is independent of CadA. These results show that in absence of CadC, *Lm* exhibits a higher BSH activity.

To assess the correlation between increased BSH activity and resistance to bile toxicity in the Δ*cadC* mutant, the survival of WT and Δ*cadC* strains was compared in BHI broth supplemented with increasing concentrations of GDCA. As previously observed,[10] GDCA inhibited the growth of WT cells in a dose-dependent manner, starting from 0.05% (Figure 1e), whereas the Δ*cadC* mutant appeared significantly more resistant to GDCA than the WT strain.

Altogether these results demonstrate that CadC has a negative impacts on *Lm* BSH activity and resistance to bile salts.

CadC indirectly represses BSH expression

CadC is a transcriptional repressor previously shown to control *cadAC* and *lspB* expression.[12] CadC-dependent *bsh* transcription was thus assessed by qRT-PCR on RNAs extracted from WT and Δ*cadC* strains grown in BHI. CadA was used as control gene whose expression is directly repressed by CadC.[12] As expected, *cadA* was highly expressed in the Δ*cadC* mutant as compared to the WT strain (Figure 2a). Similarly, *bsh* expression was significantly increased in the absence of CadC (Figure 2a), indicating that *bsh* transcription is repressed by CadC.

bsh expression was previously shown to be dependent from PrfA and σ[B] regulation.[7,10,11] In addition, we previously showed that PrfA does not control *cadC* expression.[12] To determine if the observed CadC-dependent expression of *bsh* could be due to an indirect regulation through PrfA and σ[B], the expression of these two regulators was assessed by qRT-PCR on RNAs extracted from WT and Δ*cadC* strains. Both *prfA* and *sigB*
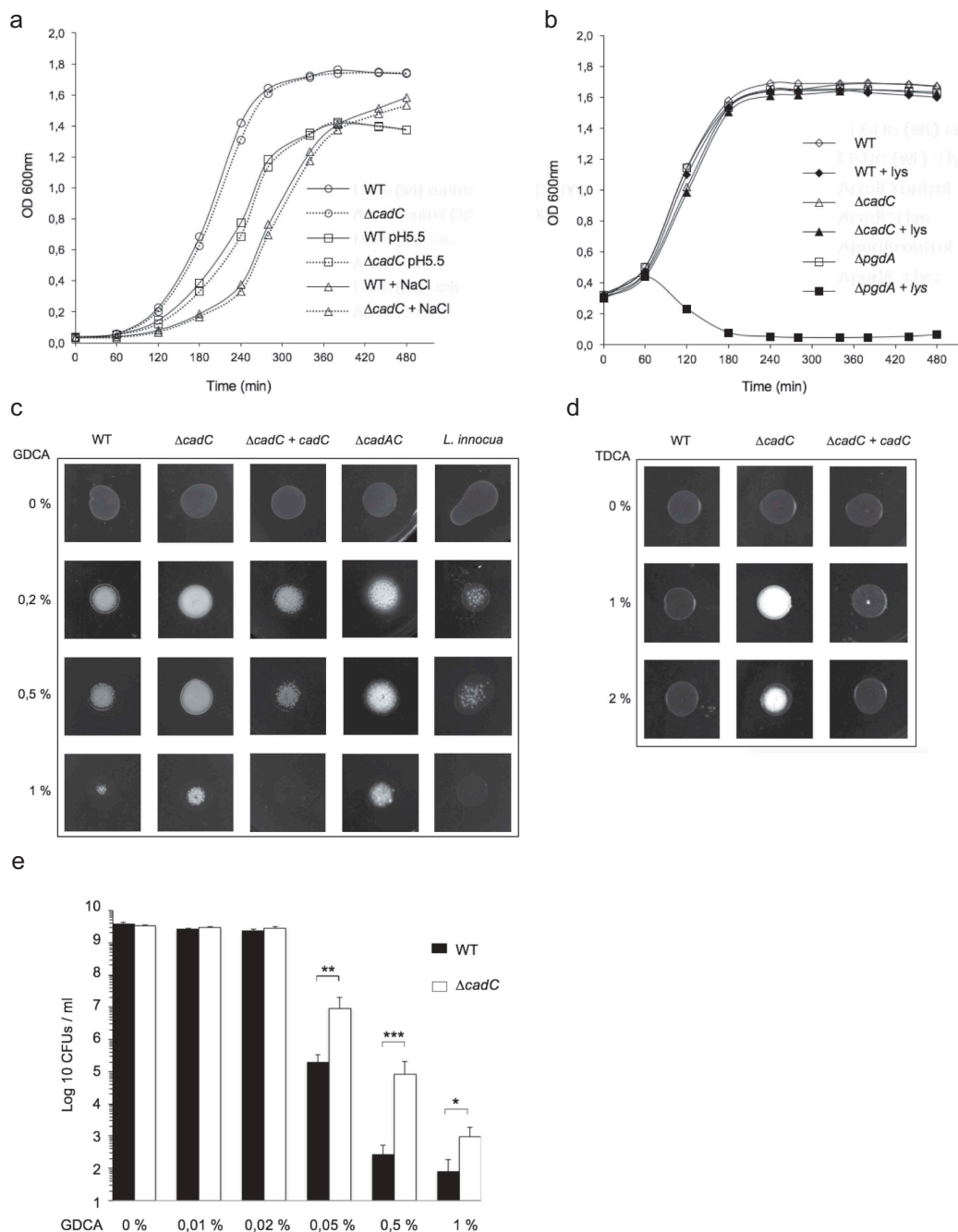
Figure 1. CadC controls BSH activity and Lm resistance to bile salts.
(A) Growth curves of WT and ΔcadC strains at 37ºC in BHI broth at pH 7, or BHI at pH 5.5 or supplemented with 5% NaCl. **(B)** Growth curves of the WT, ΔcadC and ΔpgdA strains in BHI broth supplemented with 50 µg/ml of lysozyme at 37ºC. **(C-D)** Effect of CadC on BSH activity. Lm WT, ΔcadC, ΔcadC+cadC, ΔcadAC and L. innocua were patch inoculated onto MRS agar supplemented with increasing concentrations of GDCA **(C)** or TDCA **(D)** and incubated at 37ºC for 72 h under microaerophilic conditions. BSH activity was detected by the generation of white precipitate halos of unconjugated bile acids. Experiments were performed at least twice, and representative results are shown. **(E)** Role of CadC in tolerance to bile. Overnight cultures of Lm WT and ΔcadC were inoculated into BHI supplemented with increasing concentrations of GDCA. Viable bacterial counts were performed after 16 h after by plating serial dilutions on BHI agar. Values are mean ± SD (n = 3).

appeared to be expressed independently of CadC (Figure 2a), indicating that the CadC-dependent repression of *bsh* is not achieved through PrfA or σ^B.

CadC was shown to repress the expression of target genes by direct binding to conserved CadC boxes present in their promoter.[12] Despite the absence of a CadC box in the promoter region of
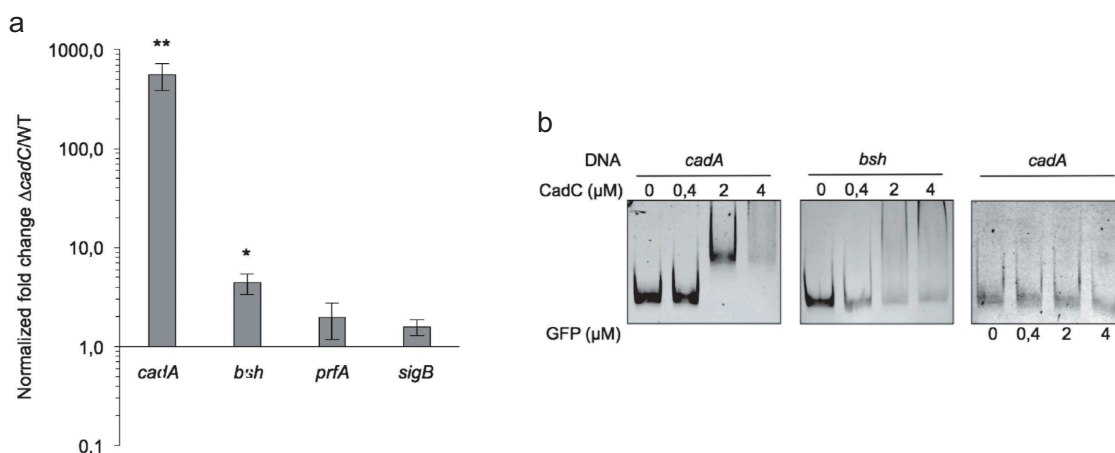
Figure 2. CadC indirectly represses *bsh* expression.
(A) Expression of *cadA, bsh, prfA* and *sigB* was analyzed by qRT-PCRs on RNAs extracted from logarithmic cultures of WT and ∆*cadC* grown in BHI at 37°C. Gene expression levels in the ∆*cadC* strain are shown as normalized to those detected in the WT. Values are mean ± SD (n = 3). * = $P < .05$, ** = $P < .01$. **(B)** Increasing amounts of purified CadC were used in electrophoretic mobility shift assays (EMSAs) with DNA fragments containing the *cadA* and *bsh* promotor regions generated by PCR using primers listed in Table S2. The promoter region of *cadA* was used as positive control and an unrelated protein (GFP) was used as negative control.

bsh, we analyzed whether *bsh* expression could be controlled by the direct binding of CadC to its promoter. Increasing amounts of purified CadC were used in EMSA with a DNA fragment containing the *bsh* promoter region (Figure 2b). The promoter region of *cadA* was used as a positive control.[12] At the CadC concentration sufficient to delay the *cadA* promoter mobility, no shift was observed for the promoter regions of *bsh*, this being also observed using higher CadC concentrations (Figure 2b). An unrelated protein (GFP) was used to verify the specificity of the *cadA*-CadC box delayed migration. This indicates that CadC do not bind directly to the *bsh* promoter region.

Altogether, these data suggest that CadC indirectly represses *bsh* expression.

CadC plays no role in Lm survival in the gastrointestinal tract

BSH was previously shown to play an important role in *Lm* persistence within the gastrointestinal tract, and an overexpression of *bsh* could generate an increased *Lm* intestinal multiplication.[7] As *bsh* is repressed by CadC, we thus analyzed the possible role of CadC in the gastrointestinal phase of listeriosis, i.e. in the earliest stage of the infectious process. We first assessed the survival/multiplication over 12-h post-inoculation of the WT and

∆*cadC* strains in the stomach of mice intragastrically inoculated with a sublethal bacterial dose (2x10⁹ CFUs). For the WT strain, the number of bacteria in mouse stomachs was around 10^6 1-h postinoculation (p.i.), and increased to reach 10^7 CFUs at 12 h, demonstrating the survival and multiplication of *Lm* in the mouse stomach environment (Figure 3a). The ∆*cadC* mutant behaved as the WT strain, excluding a role for CadC in *Lm* survival in the stomach. In addition, the persistence of the ∆*cadC* mutant was studied and compared with its parental strain in stools of mice after intragastric inoculation (2x10⁹ CFUs), over 4 d p.i. Both strains showed a regular and similar decrease in the number of viable *Lm* in mouse stools over the time (Figure 3b).

Altogether, these results demonstrate that CadC plays no critical role in *Lm* persistence within the gastrointestinal tract.

Optimal cadC-dependent regulation of BSH expression is required to confer full Lm virulence

We next evaluated the importance of the fine regulation of *bsh* expression during infection and addressed the role of CadC in this process. For this purpose, we constructed a strain in which *bsh* expression would escape the control by CadC. We replaced the *bsh* promoter by the promoter of the *iap*
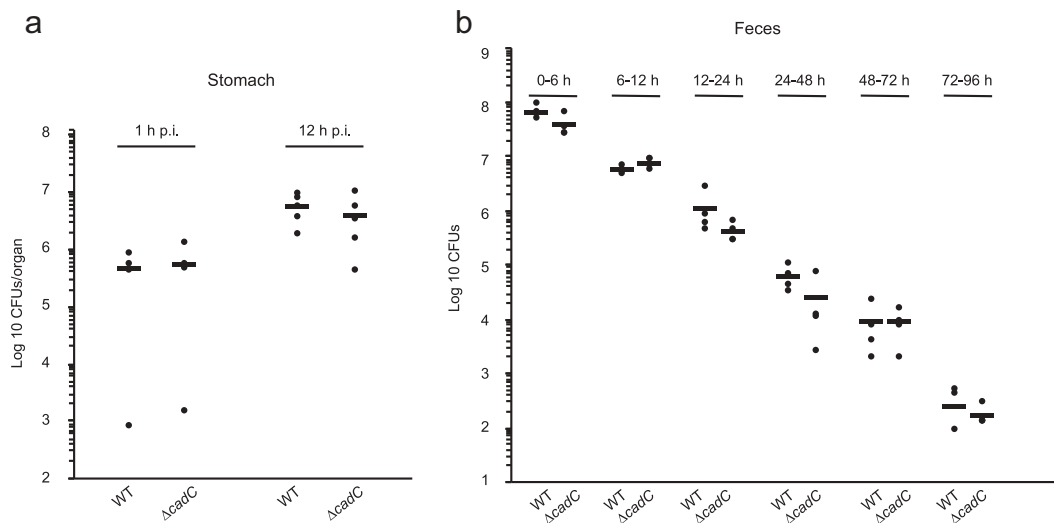
Figure 3. CadC plays no role in Lm survival in the gastrointestinal tract.
(A) Quantification of viable bacteria in mouse stomachs (n = 5) recovered 1 h and 12 h after oral inoculation with sub-lethal doses of Lm WT and ΔcadC strains. **(B)** Quantification of the fecal shedding of WT and ΔcadC strains after oral infection of mice (n = 5). Total feces produced by each animal at indicated time points were collected and processed for bacterial enumeration in *Listeria*-selective agar media. Data are presented as scatter plots, with each dot corresponding to a single animal. The mean is indicated by a horizontal line.

gene on the *Lm* chromosome (P_{iap}-*bsh*). *iap* was previously shown to have an expression pattern similar to *cadC* and inverse to *bsh*, i.e. highly expressed in infected mouse spleens as compared to growth in the intestinal lumen or in rich medium (BHI).[15,16] We first verified by EMSA that CadC does not bind to a DNA fragment corresponding to the *iap* promoter (Figure 4a). We confirmed that, in vitro, the growth of the P_{iap}-*bsh* and Δ*cadC*-P_{iap}-*bsh* strains was comparable to that of the WT (Figure 4b). CadC-independent *bsh* expression in the P_{iap}-*bsh* strain was validated by the analysis of *bsh* expression, BSH activity and resistance to bile toxicity. Whereas, as expected, *cadA* transcript levels increased in the Δ*cadC*-P_{iap}-*bsh* as compared to the P_{iap}-*bsh* strain, *bsh* expression was not significantly different in both strains (Figure 4c), confirming the CadC-independent *bsh* expression in the P_{iap}-*bsh* strain. In addition, whereas the Δ*cadC* generated a more pronounced bile acid precipitate than the WT, no differences were detectable between the Δ*cadC* and P_{iap}-*bsh* strains (Figure 4d). In accordance, as compared to *Lm* WT, both strains revealed similar phenotypes regarding resistance to bile toxicity (Figure 4e). Altogether, these results confirm that, in the P_{iap}-*bsh* strain, *bsh* is highly expressed and escapes CadC regulation.

We then tested the effect of *bsh* mis-regulation on virulence *in vivo* by intravenously challenging of mice with WT or P_{iap}-*bsh* strains (10^5 CFUs). Three days p.i., bacterial counts for P_{iap}-*bsh* were significantly lower than those for the WT in both livers and spleens, mimicking the phenotype observed for the Δ*cadC* mutant (Figure 5a). These results strongly suggest that the CadC-independent expression of *bsh* is detrimental for *Lm* throughout infection and highlight the importance of the fine-tuning of *bsh* expression for *Lm* pathogenicity.

Uncontrolled BSH expression induces MdrT overexpression in presence of bile salts

BSH is able to hydrolyze conjugated glycodeoxycholic and taurodeoxycholic acids, leading to the deconjugation of glyco- and tauro-bile acids, and the release of free cholic acids.[7,17] Host cholic acids were shown to be exported by *Lm* through the MdrT efflux pump.[8] *mdrT* is controlled by BrtA, a bile sensor which loses the ability to bind to and repress the *mdrT* promoter in the presence of cholic acid.[8,18] Interestingly, the overexpression of MdrT was shown to significantly restrict *Lm* virulence *in vivo*.[19,20] We thus hypothesized that an uncontrolled expression of *bsh in vivo* could induce an overproduction of cholic acid, in turn
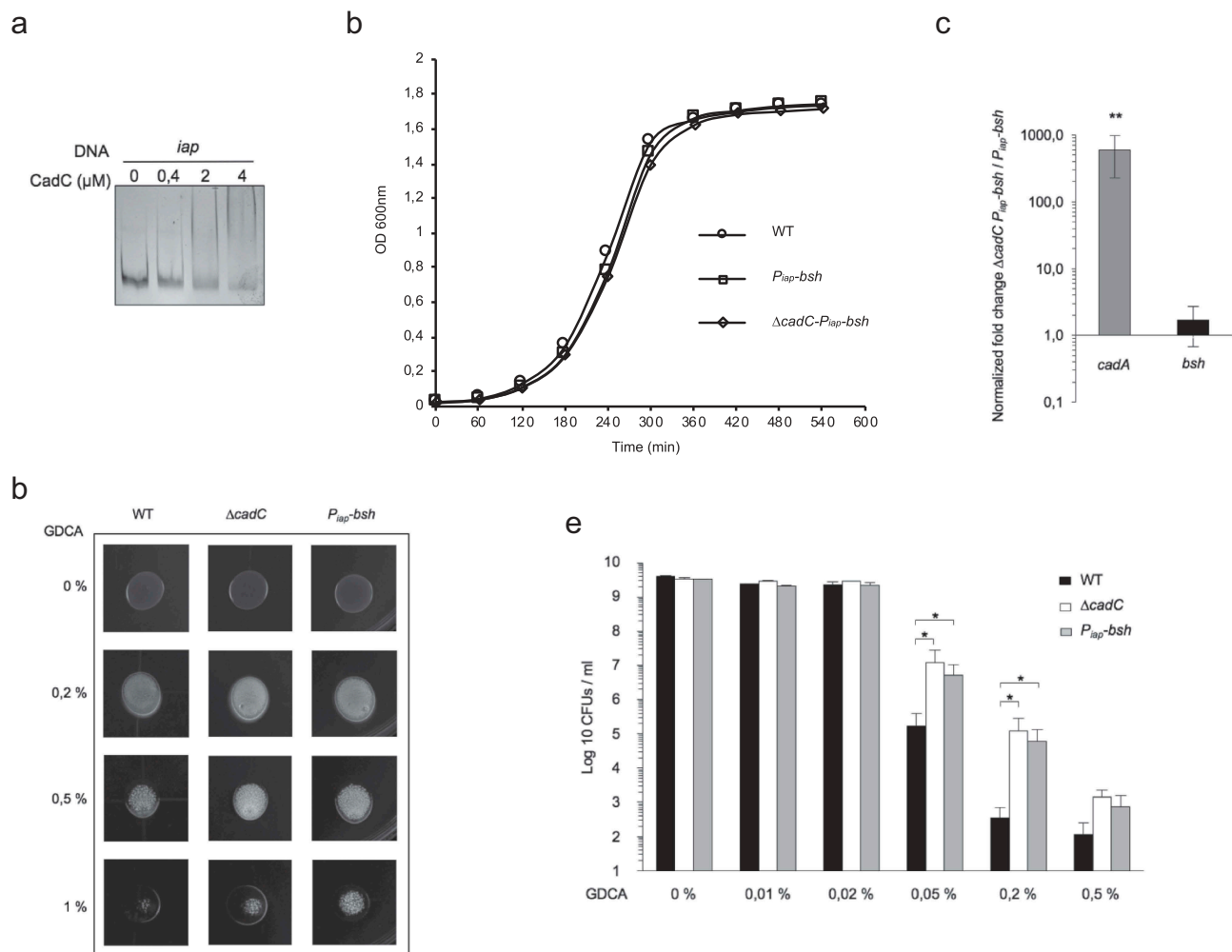
Figure 4. CadC-independent expression of *bsh* in the *P*$_{iap}$-*bsh* strain.
(A) Increasing amounts of purified CadC were used in electrophoretic mobility shift assays (EMSAs) with DNA fragments containing the *iap* promotor region generated by PCR using primers listed in Table S2. **(B)** Growth curves of the *Lm* WT, *P*$_{iap}$-*bsh* and Δ*cadC*-*P*$_{iap}$-*bsh* strains in BHI broth at 37ºC. **(C)** *cadA* and *bsh* expression was assessed by qRT-PCR on RNAs extracted from logarithmic cultures of *Lm* WT and Δ*cadC* strains in BHI at 37°C. Values are mean ± SD (n = 3). ** = $P < .01$. **(D)** *Lm* WT, Δ*cadC*, and *P*$_{iap}$-*bsh* strains were patch inoculated onto MRS agar supplemented with increasing concentrations of GDCA and incubated at 37ºC for 72 h under microaerophilic conditions. Experiments were performed at least twice, and representative results are shown. **(E)** Overnight cultures of *Lm* WT, Δ*cadC*, and *P*$_{iap}$-*bsh* strains were inoculated into BHI supplemented with increasing concentrations of GDCA. Viable bacterial counts were performed after 16 h by serial dilution and plating on BHI agar. Values are mean ± SD (n = 3). * = $P < .05$.

promoting a stronger *mdrT* expression and thus restricting *Lm* virulence.

To test this hypothesis, we analyzed by qRT-PCR the expression of *bsh* and *mdrT* in the WT and *P*$_{iap}$-*bsh* strains, in absence or presence of bile salts (GDCA). We first observed that *bsh* is more expressed in the *P*$_{iap}$-*bsh* strain as compared to the WT, independently of the presence of GDCA (Figure 5b). Whereas in absence of bile salts the uncontrolled *bsh* expression in the *P*$_{iap}$-*bsh* strain had no effect on the *mdrT* expression, the presence

of GDCA induced an increased *mdrT* expression in the *P*$_{iap}$-*bsh* strain as compared to WT (Figure 5b). *In vivo*, the lack of CadC repression and the subsequent *bsh* overexpression could thus promote *mdrT* over-expression limiting *Lm* virulence.

CadC regulates additional *Lm* genes

CadC appears thus as a crucial repressor for *Lm* infection. To assess if CadC could be a broad virulence regulator, we searched for other CadC-
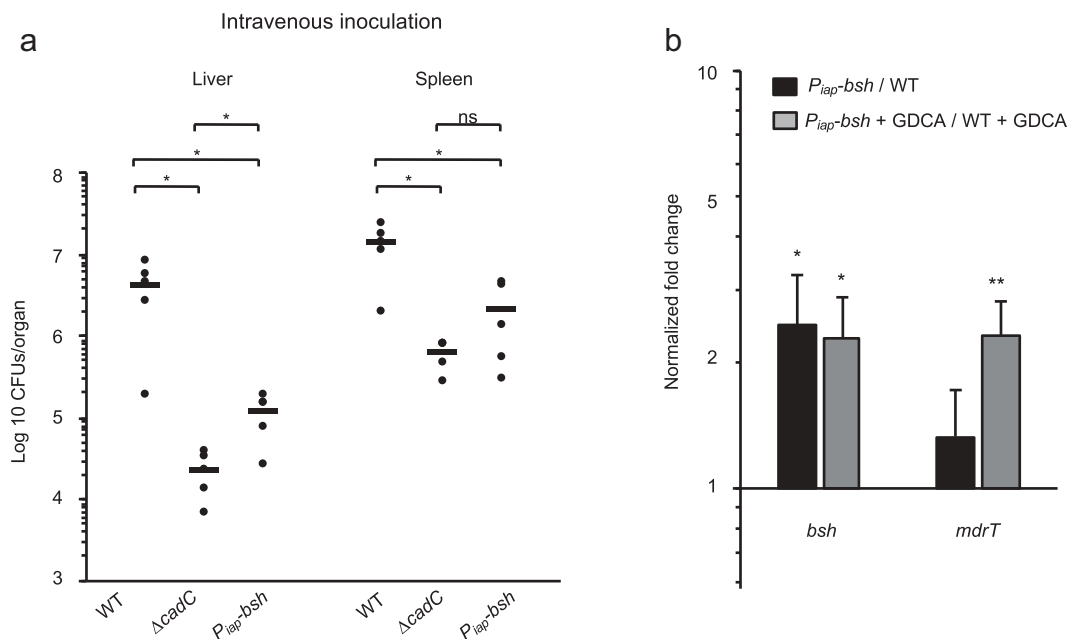
Figure 5. Uncontrolled *bsh* expression decreases Lm virulence and induces overexpression of *mdrT* in presence of bile salts. **(A)** Bacterial counts of the *Lm* WT, Δ*cadC*, and P_{iap}-*bsh* strains in the livers and spleens of mice 72 h after intravenous inoculation of 10^5 bacteria per animal. Each dot corresponds to one animal, mean values are represented by a horizontal bar. * = $P < .05$. **(B)** Expression of *bsh* and *mdrT* was assessed by qRT-PCR on RNAs extracted from logarithmic cultures of *Lm* WT and P_{iap}-*bsh* strains grown in BHI at 37°C in absence or presence of GDCA. Gene expression levels are shown as normalized to those in the WT. Values are mean ± SD (n = 3). * = $P < .05$, ** = $P < .01$.

regulated genes. The expression profile of Δ*cadC* was compared to that of WT during exponential growth in BHI at 37°C, using *Lm* tiling arrays.[16] Genes showing at least a two-fold change in their level of expression are listed in Figure 6a and Table S3. We found 53 genes differentially regulated in the Δ*cadC* mutant as compared to the WT strain, 45 of which were more expressed in absence of *cadC*. We further confirmed our results by qRT-PCR. We selected a subset of down- and up-regulated genes and performed qPCR on cDNA from bacteria grown to exponential phase. qRT-PCR results and array data exhibited a very strong correlation coefficient ($R^2 = 0.96$) (Figure 6b), validating the differential expression levels detected by transcriptomics.
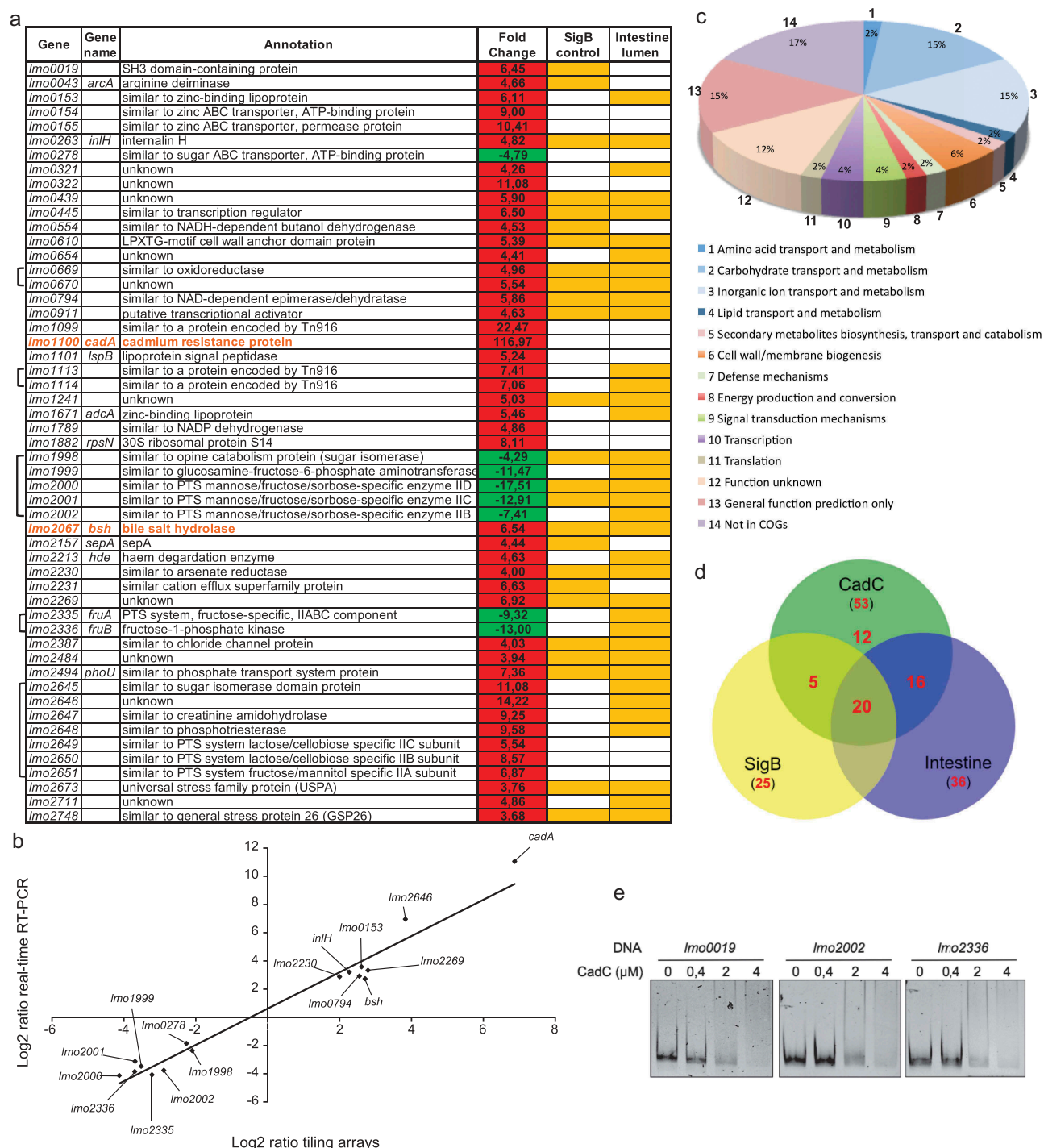
A large number of differentially expressed genes appeared to encode nutrient transport systems (Figure 6a and 6c). In particular, 8 genes are implicated in inorganic ion transport and metabolism and are up-regulated in Δ*cadC*. This group includes *cadA* and *lspB* previously shown to be directly repressed by CadC,[12] and *bsh*. Remarkably, CadC also negatively controls the expression of the LPXTG surface protein-encoding genes *inlH* and *lmo0610*, both

previously implicated in *Lm* virulence.[21,22] In addition, CadC also negatively controls the expression of *lmo2673* that encodes an universal stress protein A shown to be required for virulence.[23] Interestingly, 29 of the 45 genes more expressed in absence of CadC were previously shown to be activated during survival in the mouse intestinal lumen,[16] and 25 were shown to be activated by σ^B,[24–26] the master regulator of class II stress genes particularly important for regulating transcription during the gastrointestinal stages of *Lm* infection (Figure 6d).[27] Remarkably, 19 genes are simultaneously controlled by CadC and σ^B, and activated during the intestinal phase of the infection.

To unravel a potential mechanism of CadC transcriptional regulation, we searched for a conserved motif in the promoter region of the 53 genes. Despite an exhaustive bioinformatic analysis, we were unable to detect any common regulatory sequence. To determine whether the expression of some of these genes could be controlled by the direct binding of CadC to their promoter region, as previously described for *cadA* and *lspB*,[12] or *via* other regulatory elements,

a

Gene	Gene name	Annotation	Fold Change	SigB control	Intestine lumen
lmo0019		SH3 domain-containing protein	6,45		
lmo0043	arcA	arginine deiminase	4,66	■	
lmo0153		similar to zinc-binding lipoprotein	6,11		■
lmo0154		similar to zinc ABC transporter, ATP-binding protein	9,00		
lmo0155		similar to zinc ABC transporter, permease protein	10,41		
lmo0263	inlH	internalin H	4,82	■	
lmo0278		similar to sugar ABC transporter, ATP-binding protein	-4,79		■
lmo0321		unknown	4,26		■
lmo0322		unknown	11,08		
lmo0439		unknown	5,90	■	
lmo0445		similar to transcription regulator	6,50	■	
lmo0554		similar to NADH-dependent butanol dehydrogenase	4,53		
lmo0610		LPXTG-motif cell wall anchor domain protein	5,39	■	
lmo0654		unknown	4,41	■	
lmo0669		similar to oxidoreductase	4,96		
lmo0670		unknown	5,54		
lmo0794		similar to NAD-dependent epimerase/dehydratase	5,86		
lmo0911		putative transcriptional activator	4,63		
lmo1099		similar to a protein encoded by Tn916	22,47		
lmo1100	cadA	cadmium resistance protein	116,97		
lmo1101	lspB	lipoprotein signal peptidase	5,24		
lmo1113		similar to a protein encoded by Tn916	7,41		
lmo1114		similar to a protein encoded by Tn916	7,06		
lmo1241		unknown	5,03	■	
lmo1671	adcA	zinc-binding lipoprotein	5,46		
lmo1789		similar to NADP dehydrogenase	4,86		
lmo1882	rpsN	30S ribosomal protein S14	8,11		
lmo1998		similar to opine catabolism protein (sugar isomerase)	-4,29		
lmo1999		similar to glucosamine-fructose-6-phosphate aminotransferase	-11,47		
lmo2000		similar to PTS mannose/fructose/sorbose-specific enzyme IID	-17,51		
lmo2001		similar to PTS mannose/fructose/sorbose-specific enzyme IIC	-12,91		
lmo2002		similar to PTS mannose/fructose/sorbose-specific enzyme IIB	-7,41		
lmo2067	bsh	bile salt hydrolase	6,54		
lmo2157	sepA	sepA	4,44		■
lmo2213	hde	haem degardation enzyme	4,63		■
lmo2230		similar to arsenate reductase	4,00		■
lmo2231		similar cation efflux superfamily protein	6,63		■
lmo2269		unknown	6,92		■
lmo2335	fruA	PTS system, fructose-specific, IIABC component	-9,32		
lmo2336	fruB	fructose-1-phosphate kinase	-13,00		
lmo2387		similar to chloride channel protein	4,03		■
lmo2484		unknown	3,94		■
lmo2494	phoU	similar to phosphate transport system protein	7,36		
lmo2645		similar to sugar isomerase domain protein	11,08		
lmo2646		unknown	14,22		
lmo2647		similar to creatinine amidohydrolase	9,25		
lmo2648		similar to phosphotriesterase	9,58		
lmo2649		similar to PTS system lactose/cellobiose specific IIC subunit	5,54		■
lmo2650		similar to PTS system lactose/cellobiose specific IIB subunit	8,57		■
lmo2651		similar to PTS system fructose/mannitol specific IIA subunit	6,87		■
lmo2673		universal stress family protein (USPA)	3,76	■	
lmo2711		unknown	4,86		■
lmo2748		similar to general stress protein 26 (GSP26)	3,68	■	

c

- 1 Amino acid transport and metabolism
- 2 Carbohydrate transport and metabolism
- 3 Inorganic ion transport and metabolism
- 4 Lipid transport and metabolism
- 5 Secondary metabolites biosynthesis, transport and catabolism
- 6 Cell wall/membrane biogenesis
- 7 Defense mechanisms
- 8 Energy production and conversion
- 9 Signal transduction mechanisms
- 10 Transcription
- 11 Translation
- 12 Function unknown
- 13 General function prediction only
- 14 Not in COGs

d

b

e

Figure 6. CadC indirectly regulates additional Lm genes.

(A) *Genes* differentially expressed in the *Lm* Δ*cadC* mutant as compared to WT strain, as determined by tiling arrays. Genes whose expression is up-regulated (red) or down-regulated (green) in Δ*cadC* as compared to WT are shown. SigB regulation and expression in the intestine lumen previously described are indicated. **(B)** Validation of tiling arrays data by qRT-PCR. Fold changes in gene expression in the Δ*cadC* as compared to the WT strain, measured by tiling arrays and qRT-PCR, log transformed and compared for correlation analysis. **(C)** Relative abundance of categories of genes differentially expressed in the Δ*cadC* mutant. Colors correspond to categories in the COG database. **(D)** Venn diagram showing genes controlled by CadC, SigB or expressed in the mouse intestinal lumen. **(E)** Binding of CadC to the promoter region of regulated genes. Increasing amounts of purified CadC were used in EMSAs with PCR-generated DNA fragments containing the promoter region of genes repressed (*lmo0019*) or activated (*lmo2002* and *lmo2336*) by CadC. Experiments were performed at least twice, and representative results are shown.

increasing amounts of purified CadC were used in EMSA with DNA fragments containing the promoter region of genes up- (*lmo0019*) or down- (*lmo2002* and *lmo2336*) regulated in the Δ*cadC* mutant. At the CadC concentration that is sufficient to delay the *cadA* promoter mobility (Figure 2b), and even using higher CadC concentrations, no shift was observed for the promoter regions of the genes tested (Figure 6e).

Together our data suggest that CadC acts as a virulence gene repressor promoting bacterial dissemination during *Lm* infection.

Discussion

Bacterial adaptation to life inside the host depends on a coordinated network of activators and repressors. The presence of unrequired factors at inappropriate time frames during bacterial colonization can be detrimental to successful survival.[28] We previously showed that, during *in vivo* infection, *Lm* uses CadC to repress *lspB* expression and decrease the host inflammatory response by reducing exposure and immune recognition of the lipoprotein LpeA.[12] Here, we show that *Lm* also uses CadC to control BSH activity by repressing *bsh* expression. In accordance with its low expression in the intestinal lumen,[15,16] CadC appears dispensable for *Lm* survival in the gastrointestinal tract. Inversely, CadC is highly expressed during infection of host organs,[15,16] and CadC-dependent repression of *bsh* expression is required after crossing of the intestinal barrier to confer *Lm* full virulence. We reveal that, in presence of bile salts, an overexpression of *bsh* induces an upregulation of *mdrT*, most probably through the cholic acids (CA)-sensor BrtA. Whereas MdrT protects *Lm* from the bactericidal effects of bile,[8] its unregulated expression was shown to significantly restricts virulence *in vivo* (Figure 7).[20] This strongly suggests that the uncontrolled expression of *bsh in vivo* induces CA overproduction, in turn promoting higher *mdrT* expression and restricting *Lm* virulence. We thus propose that *Lm* uses CadC to repress *bsh* expression specifically during infection stages
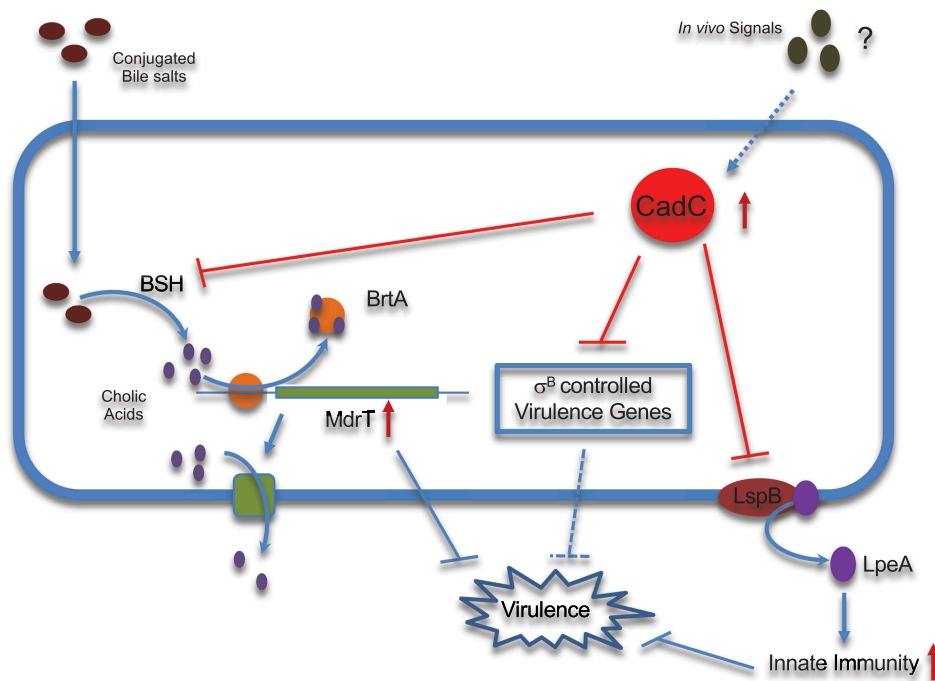
Figure 7. Model of CadC as negative regulator of *Lm* virulence genes promoting systemic infection.
Deconjugation of bile acids by BSH induces the release of free cholic acids, that are exported by *Lm* through the MdrT efflux pump. *mdrT* is controlled by BrtA, a bile sensor which loses the ability to repress *mdrT* in the presence of cholic acids. CadC represses *bsh* expression to avoid the over expression of the MdrT cholic acid efflux pump shown to restrict *Lm* virulence *in vivo*. CadC also represses *lspB* expression and diminish the host inflammatory response by reducing exposure and immune recognition of the secreted lipoprotein LpeA. CadC regulates additional genes, in particular, virulence genes activated by σ[B] during colonization of the host intestinal lumen. We propose that *Lm* CadC represses at late infection stages σ[B]-controlled genes otherwise important in the gastrointestinal tract.

where its overexpression would be deleterious for virulence. MdrT also transports c-di-AMP which is sensed by the host cytosolic innate immune receptor STING, activating a strong type I interferon (IFN-β) response.[29] Paradoxically, IFN-β production was shown to enhance susceptibility to *Lm* infection.[30,31] The diversity of physiologically relevant substrates transported by MdrT reinforces the need for its tight regulation *in vivo*, in particular through indirect CadC repression.

We identified 53 CadC-regulated genes. Despite an exhaustive bioinformatic analysis, we were unable to identify any common regulatory motif, and no CadC binding was detected on the promoter of regulated genes other than *cadAC* and *lspB*, suggesting that CadC acts indirectly to control the expression of the remaining genes. CadC appears mostly as a repressor (85% of repressed genes), ion transport and metabolism genes constituting the largest target group. *lmo0153-lmo0155 and lmo1671* encode a zinc ABC transporter and a zinc-binding protein, respectively, *lmo2494* a regulatory protein of phosphate transport, *lmo2230* an arsenate reductase and *lmo2231* a cation efflux protein. This suggests a peripheral function for CadC in controlling arsenic, zinc and phosphate homeostasis. Most importantly, three genes known to be required for *Lm* virulence, *inlH, lmo0610* and *lmo2673* are also repressed by CadC, suggesting that the *in vivo* regulation of these genes by CadC is crucial at specific infection stages. Among the few genes that appear activated by CadC, *lmo2000-lmo2002* encode components of a mannose/fructose/sorbose PTS system, and *lmo1998-lmo1999* two sugar isomerases implicated in hexosamine metabolism, converting fructose-6-phosphate into glucosamine-6-phosphate. The end product of this pathway is N-acetylglucosamine, a peptidoglycan component also used for teichoic acids decoration,[32] suggesting the involvement of CadC in the regulation of the composition and/or structure of the bacterial cell wall. However, we were unable to detect by HPLC analysis any defect in the ΔcadC cell wall. *lmo2335-lmo2336 (fruA-fruB)* operon encodes components that participate in the transport and conversion of fructose to fructose-1-6-bisphosphate, and *lmo0278* is a maltose-maltodextrin ABC transporter.[33] Fructose-1-6-bisphosphate and maltose are central metabolism intermediates, being used for glycolysis or deviated to other metabolic pathways. CadC regulation of these carbon source transporters might be important for the *Lm* growth and adaptability to the host environment, in particular the intestine.

During infection, *Lm* up-regulates the expression of major virulence regulators (PrfA, VirR), and of the master regulator of class-II stress genes, σ^B. In particular, the fine regulation of the PrfA regulon through complex PrfA-σ^B interactions appears essential during infection.[27] Indeed, bacteria need to ensure a rapid increased expression of virulence genes and their subsequent down-regulation to avoid irreversible host cell damages.[34] *cadC* is highly expressed during late infection stages,[15] and we observed here that a high proportion of CadC repressed genes were previously shown to be activated during survival in the mouse intestinal lumen,[16] and controlled by σ^B, the master transcriptional regulator of the gastrointestinal *Lm* infection stage. We propose that *Lm* CadC represses at late infection stages σ^B-controlled genes otherwise important in the gastrointestinal tract (Figure 7), revealing CadC as a crucial new player of the complex network of transcriptional regulators that contributes to fine-tune virulence gene expression over the *Listeria* infectious process. Whereas in the environment CadC is regulated by cadmium,[12] *in vivo*, during infection, it most likely responds to other signals that remain to be identified. Our study also emphasizes the importance for a pathogen of not only activating virulence genes when they are needed but also suppressing them when they are detrimental, pointing new potential therapeutic targets.

Materials and methods

Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table S1. *Listeria monocytogenes (Lm)* and *Escherichia coli (E. coli)* strains were routinely cultured aerobically at 37°C in brain heart infusion (BHI, Difco) and Lysogeny Broth (LB, Difco), respectively, with shaking. When appropriate, the following antibiotics were included in culture

media as selective agents: ampicilin (Amp), 100 µg/ml; chloramphenicol (Cm), 7 µg/ml (*Lm*) or 20 µg/ml (*E. coli*); erythromycin (Ery), 5 µg/ml.

Construction of mutant strains

Construction of the P_{iap}-*bsh* strain was performed using the splicing-by-overlap-extension (SOE) procedure. Two pairs of primers were used (*lmo2068MA-MB* and *catMA-MB*) (Table S2) to amplify a 556-bp fragment from the upstream region of *bsh* and the 684-bp *cat* gene, respectively, using Accuzyme DNA Polymerase (Bioline). Resulting products were mixed in a 1:1 ratio and reamplified using primers *lmo2068MA* and *catMB*. The final product was digested and cloned into pMAD.[35] Two other primer pairs were used (*iapMA-MB* and *bshMC-MD*) (Table S2) in PCR to amplify, respectively, the 226-bp promoter region of *iap* and the first 583-bp of *bsh*. Resulting products were mixed and reamplified using primers *iapMA* and *bshMD*. The final product was digested and cloned into pMAD already containing the first fragment. The resulting plasmid was then electroporated in *Lm* EGD-e and transformants selected at 30°C in BHI-Ery. Positive clones were re-isolated in the same medium and grown overnight at 43°C. Integrant clones were inoculated in BHI broth and grown overnight at 30°C, after which the cultures were serially diluted, plated in BHI agar and incubated overnight at 37°C. Individual colonies were tested for growth in BHI-Ery at 30°C and antibiotic-sensitive clones were screened by PCR. Plasmid constructions and mutants were confirmed by PCR and DNA sequencing.

Resistance to ph 5.5, salt stress and lysozyme

Growth under stressful stimuli was monitored as described.[36] *Lm* cultures grown overnight were appropriately diluted in BHI broth and their growth under the presence of stressful stimuli was monitored by optical density measurement at 600 nm (OD600). For comparative analysis of *Lm* resistance to pH 5.5 and salt stress, bacterial cultures were diluted 100-fold in BHI alone (control) or BHI pH 5.5 or containing 5% NaCl. To assess the *Lm* resistance to lysozyme, overnight bacterial cultures were diluted 10-fold in BHI alone (control) or BHI containing 50 µg/ml of chicken egg white lysozyme (Sigma). An *Lm* mutant strain hypersensitive to lysozyme ($\Delta pgdA$)[13] was used as a positive control for susceptibility.

BSH activity assays

Stationary cultures were dropped (10 µl) in MRS (Man, Rogosa and Sharpe) agar plates supplemented with increasing concentrations (0.2%, 0.5%, 1% and 2%) of purified glycochenodeoxycholic acid (GDCA, Merck Millipore) or taurochenodeoxycholic acid (TDCA, Santa Cruz Biotechnologies). Plates were incubated anaerobically for 72 h at 37°C (GENbox, Biomérieux).

Sensitivity to bile salts

Lm strains were grown to log phase in BHI broth at 37°C. Cultures were diluted in BHI and 5×10^3 bacteria/ml were challenged with increasing concentrations (0.01%, 0.02%, 0.05%, 0.5% and 1%) of GDCA in a 24-well plate. Plates were then incubated with agitation at 37°C in aerobic conditions. After 16 h, CFUs were assessed by bacterial enumeration of serial dilutions on BHI agar.

RNA techniques

Lm cultures were grown in BHI to exponential phase (OD$_{600 \text{ nm}}$ = 0.8) and total RNA isolated by the phenol-chloroform method described elsewhere,[37] with modifications as described next. After lysis, RNA purification were performed using the TripleXtractor reagent (Grisp) following the manufacturer's recommendations. DNA was eliminated by DNase treatment (Turbo DNA-free, Ambion) and RNA purity and integrity was verified by 1% (w/v) agarose gel electrophoresis and Experion Automated Electrophoresis System (Bio-Rad Laboratories). One µg of RNA was reverse-transcribed into cDNA using a random hexamer cocktail-based kit (iScript Kit, Bio-Rad Laboratories). qPCR was performed on one µg of cDNA in a 20-µl reaction volume using the iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories) and a real-time PCR detection system (iQ5, Bio-Rad Laboratories) with the following cycling protocol: 1 cycle at 95°C (3 min); 40 cycles at

95°C (10 s), 56°C (20 s) and 72°C (20 s). Primers are listed in Table S2. Each group comprises three biological replicates each with three technical replicates. Data were normalized to that of a reference housekeeping gene (16S rRNA) and analyzed by the comparative threshold (ΔΔCt) method.

Electrophoretic mobility shift assays (EMSAs)

Protein-DNA binding was set up in 20 μl reactions containing 100 ng of DNA synthesized with primers described in Table S2, binding buffer (50 mM Tris-HCl pH7.4, 6 mM MgCl$_2$, 100 mM NaCl, 50 mM KCl) and increasing amounts of purified proteins (CadC/GFP) as previously described.[12] DNA was first incubated in binding buffer for 5 min followed by gentle mixing of the protein and 20 min incubation at room temperature. The total reaction was loaded into a 10% acrylamide native gel and ran in TAE buffer. The gel was stained for 10 min in a 0.01% GreenSafe Premium (NZYTech) TAE buffer solution and imaged in a GelDoc XR+ System (Bio-Rad Laboratories).

In vivo infection studies

Animal infections were performed on 6- to 9-week-old specific pathogen-free female C57BL/6 mice (Charles River Laboratories) maintained at the IBMC animal facilities, in high-efficiency particulate air (HEPA) filter-bearing cages under 12-h light cycles and in an *ad libitum* regiment of sterile chow and autoclaved water. Intravenous infections were performed by inoculation of 10^5 CFUs through tail vein injection as described.[38,39] For oral infections mice were starved for 8–12 h before the procedure and inoculated with 2×10^9 CFUs (in PBS with 150 mg/ml CaCO$_3$) by gavage. Mice were sacrificed by general anesthesia at indicated time points. Stomach, spleen and liver of each animal were aseptically removed, homogenized in PBS and homogenates were serially diluted and plated on BHI-agar plates. For analysis of *Lm* fecal carriage, total feces produced by each infected animal (n = 5 per strain) up to a given time-point were collected, homogenized in PBS and serial dilutions were plated in *Listeria* selective agar media (Oxoid) for bacterial enumeration. Animal procedures followed the guidelines of the

European Commission for the handling of laboratory animals (directive 2010/63/EU), the Portuguese legislation for the use of animals for scientific purposes (Decreto-Lei 113/2013), and were approved by the IBMC Animal Ethics Committee as well as by the Direcção Geral de Alimentação e Veterinária, the Portuguese authority for animal protection, under license 015302.

Expression tiling arrays

ListIP Tiling Arrays were used.[16] RNAs were reverse-transcribed using SuperScript II reverse transcriptase (Life Technologies). cDNA was digested by DNase I (Turbo DNA-free, Ambion) and the size of digestion products was analyzed in the Agilent Bioanalyser 2100. Sample preparation for each chip was then processed following the Affymetrix GeneChip Expression Analysis Technical Manual (P/N 702232 Rev. 2) as previously described.[16] Scanning of the arrays was then performed using the GeneChip scanner 3000. Intensity signals of each probe cells were computed by the GeneChip operating software (GCOS). Data analysis of the tiling sub-array was performed using the Bioconductor software (http://www.bioconductor.org) based on R package as described in.[16]

Statistics

Statistics were performed with Prism (GraphPad), using unpaired two-tailed Student's *t*-test to compare means of two groups, and one-way ANOVA with Tukey's post-hoc test for pairwise comparison of means from more than two groups, or with Dunnett's post-hoc test for comparison of means relative to the mean of a control group.

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Author contributions

Conceptualization (Design), S.S. and D.C.; Investigation (Experimental work), A.V., R.P., A.C. and C.A. Writing – Original Draft, D.C.; Writing – Review & Editing, A.V., R.P.,

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Disclosure of potential conflict of interest

No potential conflict of interest were disclosed.

Data availability

All data needed to evaluate the conclusions in the paper are present in the paper or in the supplementary information. Expression tiling array results are available at the ArrayExpress database (https://www.ebi.ac.uk/arrayexpress/) under the accession number E-MTAB-7857.

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